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Formation of Reactive Cyclopentenone Compounds *in Vivo* as Products of the Isoprostane Pathway*

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Cyclopentenone prostaglandins A_2 and J_2 are reactive compounds that possess unique biological activities. However, the extent to which they are formed *in vivo* remains unclear. In this study, we explored whether D_2/E_2-isoprostanes undergo dehydration *in vivo* to form A_2/J_2-isoprostanes. Oxidation of arachidonic acid *in vitro* generated a series of compounds that were confirmed to be A_2/J_2-isoprostanes by mass spectrometric analyses. A_2/J_2-isoprostanes were detected *in vivo* esterified to lipids in livers from normal rats at a level of 5.1 ± 2.3 ng/g, and levels increased dramatically by a mean of 24-fold following administration of CCl_4. An A_2-isoprostane, 15-A_{2t}-isoprostane, was obtained and found to readily undergo Michael addition with glutathione and to adduct covalently to protein. A_2/J_2-isoprostanes could not be detected in the circulation, even following CCl_4 administration, which we hypothesized might be explained by rapid formation of adducts. This was supported by finding that essentially all the radioactivity excreted into the urine following infusion of radiolabeled 15-A_{2t}-isoprostane into a human volunteer was in the form of a polar conjugate(s). These data identify a new class of reactive compounds that are produced *in vivo* as products of the isoprostane pathway that can exert biological effects relevant to the pathobiology of oxidant injury.

Cyclopentenone (CP)[1] prostaglandins (PG) of the A and J series have been shown to be produced *in vitro* by dehydration of the cyclopentane ring of PGE_2 and PGD_2, respectively. These compounds have attracted considerable attention because they exert unique biological actions. CP-PGs are actively incorporated into cells and accumulate in the nucleus (1, 2). They have been shown to inhibit cellular proliferation with a G_1 cell cycle arrest and to induce differentiation, an effect that may be related to their ability to modulate a variety of growth-related and stress-induced genes (3–5). These cytostatic effects can be reversible, but higher concentrations are cytotoxic and induce apoptosis (4, 6, 7). Interestingly, at very low concentrations, PGA was found to stimulate cellular proliferation (8). CP-PGs can also activate nuclear peroxisome proliferator-activated receptor-γ and suppress macrophage activation and inflammatory responses (9–11). Furthermore, CP-PGs exhibit antiviral activity (12). The common feature in these compounds is the presence of a reactive α,β-unsaturated carbonyl group, which is very susceptible to nucleophilic addition reactions and seems to be essential for many of their biological effects (13–15).

Although the biological effects exerted by CP-PGs have been studied in some detail, the extent to which they are formed *in vivo* has been the subject of continuing controversy for over 2 decades (16–18). Fueling this controversy has always been the uncertainty as to what extent dehydration of PGE_2 and PGD_2 *ex vivo* during sample processing contributes to the amount of PGA_2 and PGJ_2 detected. Recently, Δ^{12}-PGJ_2 was definitively identified in human urine by Hayaishi and co-workers (19). However, the amounts in urine from males were >2-fold higher than the amounts in urine from females. This is difficult to reconcile with the evidence suggesting that there is no sexual difference in the amount of PGD_2 produced *in vivo* in humans (20). Convincing evidence was presented that the Δ^{12}-PGJ_2 detected in urine unlikely arose as a result of dehydration of urinary PGD_2 *ex vivo* during sample processing. However, it is difficult to know to what extent PGD_2 may undergo dehydration in the genitourinary tract prior to voiding. This is of particular interest since the same authors recently reported that high levels of PGD synthase are present in human male reproductive organs and that seminal plasma greatly facilitates dehydration of PGD_2 (21). Furthermore, they also recently reported that the level of PGD synthase in male urine is approximately twice that found in female urine (22). Taken together, these findings suggest that at least some of the Δ^{12}-PGJ_2 detected in urine may have arisen from dehydration of PGD_2 in the genitourinary tract and may explain the higher levels of Δ^{12}-PGJ_2 in urine from males. This does not confute the occurrence of Δ^{12}-PGJ_2 in human urine, but only raises the question of its origin, that being whether it arose from systemic sources or from local production in the genitourinary tract. Therefore, it still remains unclear whether CP-PGs are ubiquitously produced throughout the body.

Free radicals have been increasingly implicated in the pathogenesis of a wide spectrum of human disease processes (23, 24). Previously, we reported the discovery of PG-like compounds, now termed isoprostanes (IsoPs), that are produced *in vivo* nonenzymatically as products of free radical-induced peroxidation of arachidonoyl lipids (25, 26). Our initial report described the formation of PGF_2-like compounds (F_2-IsoPs). Subsequently, we reported the finding that PGD_2- and PGE_2-like compounds (D_2/E_2-IsoPs) are also produced in abundance *in vivo* (27). In the studies reported herein, we explored whether D_2/E_2-IsoPs undergo dehydration *in vivo* to produce CP-IsoPs (A_2/J_2-IsoPs). Our interest in this stems from the fact that such compounds may be identified as a new class of reactive compounds that are formed as products of free radical-induced lipid peroxidation that exert unique biological actions relevant to the pathobiology of oxidative stress.

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[1] The abbreviations used are: CP, cyclopentenone; PG, prostaglandin; IsoP, isoprostane; HPLC, high pressure liquid chromatography; GC, gas chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; PFB, pentafluorobenzyl; TMS, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

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EXPERIMENTAL PROCEDURES

Conversion of 15-E_{2t}-IsoP to 15-A_{2t}-IsoP—8-Iso-[^{3}H]PGA_2, termed 15-A_{2t}-IsoP according to the approved nomenclature for IsoPs (28), was obtained by treatment of 15-E_{2t}-[^{3}H]IsoP (8-iso-PGE_2) (Cayman Chemical Co., Inc., Ann Arbor, MI) with 0.1 N HCl (29, 30), purified by normal phase HPLC, and analyzed by NMR for structural confirmation.

Analysis of A_2/J_2-IsoPs—A_2/J_2-IsoPs were analyzed by GC/NICI/MS using a modification of methods described for the analysis of other IsoPs (31). Briefly, 10 ng of [$^2H_4$]PGA_2 was added to samples as an internal standard, after which compounds were partially purified using C_{18} and Silica Sep-Pak cartridges (Waters), methoximated, and converted to pentafluorobenzyl (PFB) esters. Compounds were then purified by chromatography on silica 60ALK6D TLC plates (Whatman) using a solvent system of hexane/acetone (70:30, v/v). Compounds migrating in the region from 0.5 cm below the PFB ester of PGJ_2 to 1 cm above the PFB ester of PGA_2 were scraped, extracted with ethyl acetate, converted to a trimethylsilyl (TMS) ether derivative, and then analyzed by GC/NICI/MS. The major ions generated in the NICI mass spectra of the PFB ester, *O*-methyloxime, TMS ether derivative of A_2/J_2-IsoPs and [$^2H_4$]PGA_2 are the carboxylate anions at *m/z* 434 and *m/z* 438, respectively. Quantitation was performed by integration of the peak areas.

Analysis of A_2/J_2-IsoPs as a Piperidyl-enol-trimethylsilyl Ether Derivative—Treatment of PGA with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and piperidine has been shown to convert it to a piperidyl-*enol*-TMS ether derivative, which is specific for A-ring prostanoids (32). Thus, we analyzed for the formation of this derivative with CP-IsoPs. PFB esters of putative CP-IsoPs were treated with a 1:1 mixture of BSTFA/piperidine for 1 h at 60 °C and analyzed by GC/NICI/MS monitoring of the carboxylate anions at *m/z* 562 for the IsoPs and *m/z* 566 for [$^2H_4$]PGA_2.

Analysis of A_2/J_2-IsoPs by GC/Electron Ionization/MS—Putative A_2/J_2-IsoPs generated during oxidation of arachidonic acid *in vitro* were subjected to purification using two HPLC systems: (*a*) normal-phase HPLC on a 5-μm Econosil SI column using an isocratic solvent system of hexane/isopropyl alcohol/acetic acid (97:3:0.1, v/v/v) and (*b*) reversed-phase HPLC on a 5-μm Econosil C_{18} column using an isocratic solvent system of acetonitrile/water/acetic acid (38:62:0.1, v/v/v). 15-A_{2t}-[^{3}H]IsoP was added prior to HPLC purification, and compounds that coeluted with 15-A_{2t}-[^{3}H]IsoP were then converted to a PFB ester, *O*-methyloxime, TMS ether derivative and analyzed by GC/electron ionization/MS.

Analysis of D_2/E_2-IsoPs by GC/NICI/MS—Analysis of D_2/E_2-IsoPs by GC/NICI/MS was performed as described (27). The amounts of D_2/E_2-IsoPs reported in this study differ from those in our previous reports (27). In our previous reports, quantification was based on the intensity of a single prominent peak, whereas in this study, the integrated area under all peaks was used for quantification.

Oxidation of Arachidonic Acid in Vitro—Non-esterified arachidonic acid was dissolved in ethanol and then oxidized in 50 mM Tris buffer for 45 min using an iron/ADP/ascorbate oxidizing system as described (33). 1-Palmitoyl-2-arachidonoyl-phosphatidylcholine was prepared and oxidized in an identical fashion as non-esterified arachidonic acid at a concentration equivalent to 1 mg/ml arachidonic acid. After oxidation, the phospholipid was extracted and hydrolyzed as described below for liver phospholipids.

In Vivo Model of Free Radical-induced Liver Injury—Free radical-induced lipid peroxidation in liver was induced in rats by administration of CCl_4 (25). Two hours after treatment, animals were killed. Livers were then removed, snap-frozen in liquid N_2, and either processed immediately or stored at −70 °C.

Extraction, Purification, and Hydrolysis of Phospholipids—Lipids from livers of CCl_4-treated and control rats were extracted as described (31). The lipid extracts were then hydrolyzed enzymatically using *Apis mellifera* bee venom phospholipase A_2 (27).

Conjugation of 15-A_{2t}-IsoP and PGA_2 with GSH in Vitro—Radiolabeled 15-A_{2t}-IsoP and PGA_2 were incubated at 37 °C with GSH in phosphate buffer (pH 6.5) in the presence of 19 units/ml glutathione *S*-transferase (Sigma). The molar ratio of the prostanoids to GSH was 1:10. At the designated time points, aliquots were removed, acidified immediately to pH 3, and extracted twice with 2 volumes of methylene chloride. The formation of a GSH conjugate was assessed by determining the percent of radioactivity that did not extract into organic solvent (14). We predetermined that 93 ± 4% (mean ± S.E.) of PGA extracts into methylene chloride from buffer solutions at pH 3. Control incubations were carried out in the absence of GSH and enzyme.

Formation of 15-A_{2t}-IsoP and PGA_2 Covalent Adducts with Albumin—Radiolabeled PGA_2 and 15-A_{2t}-IsoP were incubated at 37 °C with 20 mg/ml human serum albumin in phosphate buffer (pH 7.4). The molar ratio of prostanoids to albumin was 1:20. At the designated time points, aliquots were withdrawn, and 10 volumes of cold ethanol were immediately added to precipitate the albumin. The formation of covalent albumin adducts was assessed by determining the percent of radioactivity present in the albumin precipitate after centrifugation. Control incubations were carried out in the absence of albumin.

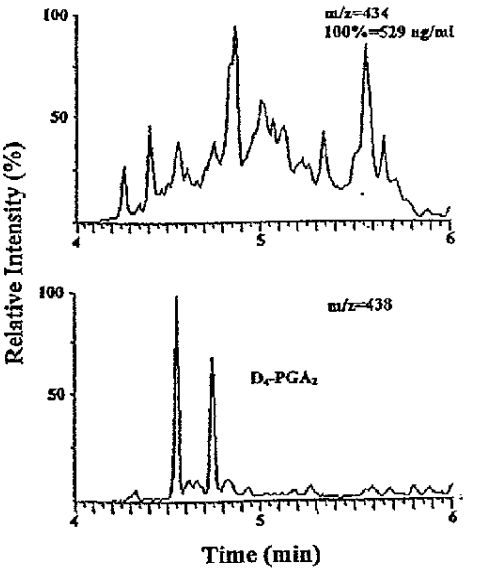

FIG. 1. **Selected ion current chromatograms from the analysis of the formation of A_2/J_2-IsoPs during oxidation of arachidonic acid *in vitro*.** The peaks in the *m/z* 438 ion current chromatogram represent the *syn*- and *anti*-*O*-methyloxime isomers of the [$^2H_4$]PGA_2 internal standard. In the *m/z* 434 chromatogram are a series of peaks consistent with the presence of A_2/J_2-IsoPs. The summed total amount of the putative A_2/J_2-IsoPs formed was 529 ng/mg of arachidonic acid.

Formation of Polar Conjugates of 15-A_{2t}-IsoP in Humans—After informed consent was obtained, a small tracer quantity (1 μCi) of 15-A_{2t}-[^{3}H]IsoP (150 Ci/mmol) was dissolved in 200 μl of ethanol, diluted to 10 ml with sterile saline, and infused over 10 min into the antecubital vein of a normal male volunteer. Individual voided urine specimens were collected over a period of 10 h and assayed for radioactivity. The formation of polar conjugates was assessed by determining the percent of radioactivity in a 1-ml aliquot of urine that did not extract into 2 volumes of methylene chloride at pH 3.

RESULTS

Initially, we explored whether A_2/J_2-IsoPs are formed during oxidation of arachidonic acid *in vitro* (Fig. 1). In the upper *m/z* 434 chromatogram are multiple peaks with a similar retention time as the *m/z* 438 peaks representing the *syn*- and *anti*-*O*-methyloxime isomers of [$^2H_4$]PGA_2 (lower chromatogram), consistent with the formation of CP-IsoPs. The amount of the compounds formed was 529 ± 135 ng/mg of arachidonic acid (mean ± S.E., n = 4).

Additional analyses further supported the identity of these compounds as A_2/J_2-IsoPs. First, no *m/z* 433 peaks were present, indicating that the *m/z* 434 peaks were not natural isotope peaks of compounds generating an ion less than *m/z* 434. When analyzed as [$^2H_9$]TMS ether and *O*-[$^2H_3$]methyloxime derivatives, all of the original *m/z* 434 peaks disappeared, and an identical pattern of new peaks appeared 9 and 3 Da higher, respectively (data not shown), indicating that all of the compounds had one hydroxyl and one carbonyl group. When analyzed following catalytic hydrogenation, there was a disappearance of the *m/z* 434 peaks and the appearance of new intense peaks 6 Da higher at *m/z* 440 (Fig. 2). No peaks were detected at *m/z* 436, 438, or 442. This indicated that the compounds

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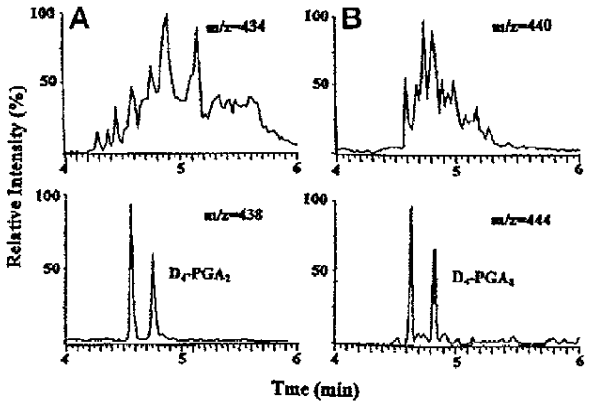

FIG. 2. **Analysis of the putative A_2/J_2-IsoPs formed during oxidation of arachidonic acid *in vitro* prior to and after catalytic hydrogenation.** *A*, analysis of compounds prior to hydrogenation. The peaks in the *m/z* 434 ion current chromatogram represent putative A_2/J_2-IsoPs, and the peaks in the *m/z* 438 chromatogram represent the [$^2H_4$]PGA$_2$ internal standard. No compounds were detected 6 Da above *m/z* 434 at *m/z* 440 prior to hydrogenation (not shown). *B*, analysis of compounds following hydrogenation. Both the internal standard and the *m/z* 434 peaks in *A* have shifted upwards 6 Da following hydrogenation, indicating the presence of three double bonds.

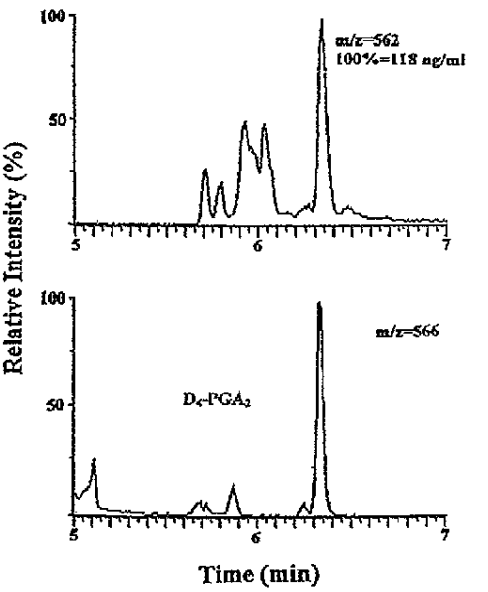

FIG. 3. **Analysis of A_2/J_2-IsoPs generated during oxidation of arachidonic acid *in vitro* as a PFB ester, piperidyl-*enol*-TMS ether derivative.** The peaks in the *m/z* 566 chromatogram represent the [$^2H_4$]PGA$_2$ internal standard. In the *m/z* 562 chromatogram are a series of peaks consistent with the formation of a piperidyl-*enol*-TMS ether derivative of CP-IsoPs.

contained three double bonds. In addition, analysis following treatment with BSTFA/piperidine resulted in the formation of a piperidyl-*enol*-TMS ether derivative (Fig. 3). The amount of A_2/J_2-IsoPs analyzed as this derivative was calculated to be 118 ± 32 ng/mg of arachidonic acid (mean ± S.E., $n = 4$), which is less than the amount formed when analyzed as an *O*-methyloxime, TMS ether derivative. This discrepancy can be explained by the fact that we have found that, whereas treatment of PGA$_2$ with BSTFA/piperidine efficiently converts it to the piperidyl-*enol*-TMS ether derivative, only trivial amounts of this derivative are formed with PGJ$_2$. By analogy, therefore, only a portion of the mixture of A_2/J_2-IsoPs would be expected to form this derivative.

Further evidence for the identity of these compounds as CP-IsoPs was obtained by analyzing the compounds by electron ionization/MS to obtain complete mass spectra. Compounds were analyzed as a PFB ester, *O*-methyloxime, TMS ether derivative. One of the mass spectra obtained is shown in Fig. 4. As noted, a molecular ion is present at *m/z* 615. Other prominent ions are present at *m/z* 600 (M − 15, loss of ·CH_3), *m/z* 584 (M − 31, loss of ·OCH_3), *m/z* 544 (M − 71, loss of ·$CH_2(CH_2)_3CH_3$), *m/z* 525 (M − 90, loss of Me_3SiOH), *m/z* 494 (M − (90 + 31)), *m/z* 308 (M − 307, loss of ·$CH_2CH{=}CH(CH_2)_3COOC_6F_5$), *m/z* 277 (M − (307 + 31)), *m/z* 218 (M − (307 + 90)), *m/z* 199 ($^+CH{=}CH{-}CH(Me_3SiOH)(CH_2)_4CH_3$), *m/z* 181 (base) ($^+CH_2C_6F_5$), and *m/z* 173 ($Me_3SiO^+{=}CH(CH_2)_4CH_3$). These ions or analogous ions and losses of other derivatives are also intense ions in the mass spectra of CP-PGs (34),[2] indicating that this was a mass spectrum of a 15-series CP-IsoP in which the side chain hydroxyl is located at C-15. Although the structure depicted in Fig. 4 is an A-ring IsoP, ions are not present that would allow a differentiation between an A-ring and a J-ring compound.

We then explored whether CP-IsoPs are also formed *in vivo*. Previously, we showed that IsoPs are initially formed *in situ* esterified in tissue phospholipids (26). Therefore, we assayed for the presence of A_2/J_2-IsoPs esterified in livers from rats that had been treated with CCl_4 to induce lipid peroxidation (Fig. 5*A*). Again, a series of *m/z* 434 peaks were present similar to what was found following oxidation of arachidonic acid *in vitro*, although the pattern of the peaks differed somewhat. This may be explained by our observation that there appears to be a steric influence of phospholipids on the formation of different IsoP isomers. In support of this notion, the pattern of peaks present following oxidation of arachidonoylphosphatidylcholine *in vitro* was found to be very similar to the pattern of peaks detected in esterified lipids in liver (Fig. 5*B*).

Further evidence for the identity of these compounds generated *in vivo* as A_2/J_2-IsoPs was obtained utilizing similar approaches used for the structural identification of the compounds formed *in vitro*. Analysis as a [$^2H_9$]TMS ether derivative, an *O*-[$^2H_3$]methyloxime derivative, and following catalytic hydrogenation indicated that all of the compounds had one hydroxyl group, one carbonyl, and three double bonds (data not shown). Furthermore, the compounds formed a piperidyl-*enol*-TMS ether derivative (Fig. 6), and the pattern of peaks was almost identical to that formed by the CP-IsoPs generated *in vitro* (Fig. 3), although the relative abundance of individual peaks differed somewhat.

We then compared the relative amounts of A_2/J_2-IsoPs and D_2/E_2-IsoPs present in livers from both normal and CCl_4-treated rats to determine the extent to which D_2/E_2-IsoPs undergo dehydration *in vivo* (Fig. 7). Of interest was the finding that A_2/J_2-IsoPs could be detected esterified in the livers of normal rats at a level of 5.1 ng/g of liver. Relative to the amounts of D_2/E_2-IsoPs measured, A_2/J_2-IsoPs were present at levels indicating that the extent to which D_2/E_2-IsoPs undergo dehydration *in vivo* is not inconsequential. Following administration of CCl_4, the levels of both A_2/J_2-IsoPs and D_2/E_2-IsoPs increased dramatically and to a similar extent by a mean of 23.9- and 21.2-fold, respectively.

We then carried out experiments to exclude the possibility that dehydration of D_2/E_2-IsoPs *ex vivo* during sample processing contributed significantly to the levels measured in liver. That this was not the case was supported by the finding that <1% of either PGE$_2$ or PGD$_2$ that was added to a lipid extract

[2] Y. Chen, W. E. Zackert, L. J. Roberts II, and J. D. Morow unpublished data.

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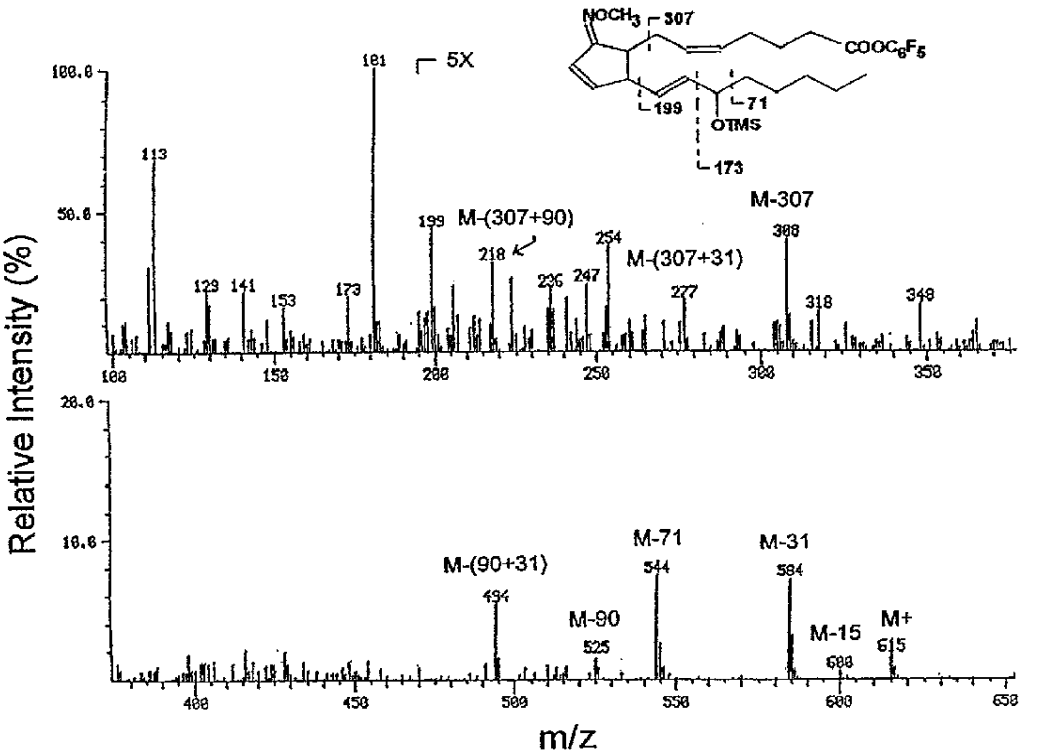

FIG. 4. **Representative electron ionization mass spectrum obtained from the analysis of A_2/J_2-IsoPs generated from oxidation of arachidonic acid *in vitro* as a PFB ester, *O*-methyloxime, TMS ether derivative.** See "Results" for interpretation of the mass spectrum.

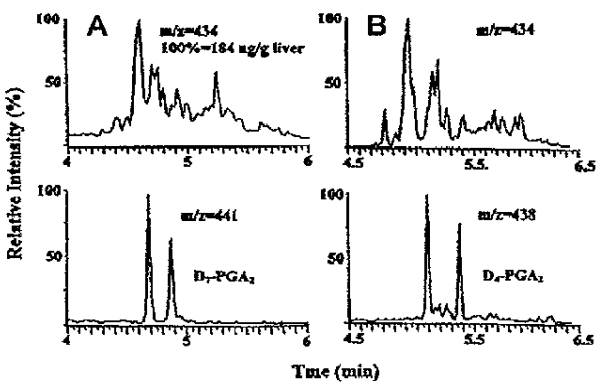

FIG. 5. *A*, analysis of A_2/J_2-IsoPs formed *in vivo* esterified in lipids in the liver of a rat treated with CCl_4. The peaks in the *m/z* 441 ion current chromatogram represent the $[^2H_4]PGA_2$ internal standard. In the *m/z* 434 ion current chromatogram are a series of peaks consistent with the presence of A_2/J_2-IsoPs. *B*, analysis of A_2/J_2-IsoPs following oxidation of arachidonoyl-phosphatidylcholine *in vitro*. In the *m/z* 434 chromatogram is a series of peaks consistent with the presence of A_2/J_2-IsoPs in a pattern that is very similar to the pattern of peaks detected in rat liver.

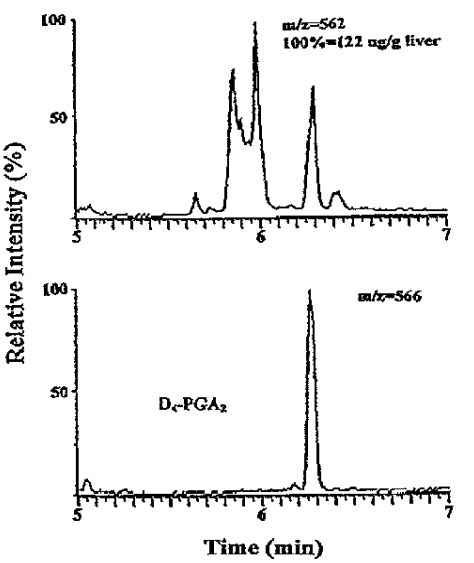

FIG. 6. **Analysis of CP-IsoPs esterified in the liver of a rat treated with CCl_4 as a PFB ester, piperidyl-*enol*-TMS ether derivative.** The peaks in the *m/z* 562 ion current chromatogram represent CP-IsoPs, and the *m/z* 566 peaks represent the $[^2H_4]PGA_2$ internal standard.

of liver and then processed and analyzed had undergone dehydration to form PGA_2 and PGJ_2, respectively.

Our major interest in the possibility that CP-IsoPs may be formed *in vivo* is because these compounds should be reactive molecules that are susceptible to nucleophilic addition reactions. Therefore, we compared the ability of one of the CP-IsoPs, 15-A_{2t}-IsoP, and PGA_2 to conjugate with GSH and adduct to protein, using albumin as a model. We recently showed that one of the E_2-IsoPs that is produced *in vivo* is 15-E_{2t}-IsoP (25). Its dehydration product, 15-A_{2t}-IsoP should therefore also be one of the CP-IsoPs that is produced *in vivo*. We initially determined the time course of conjugation of GSH with radiolabeled 15-A_{2t}-IsoP and PGA_2 in the presence of glutathione *S*-transferase. Formation of GSH conjugates was assessed by

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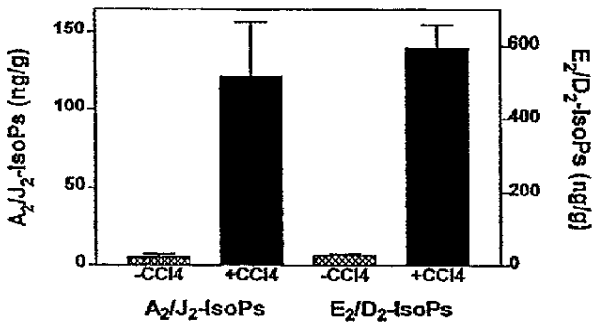

FIG. 7. **Analysis of CP-IsoPs and D_2/E_2-IsoPs esterified in livers from normal rats and following administration of CCl_4 to induce an oxidant injury to the liver.**

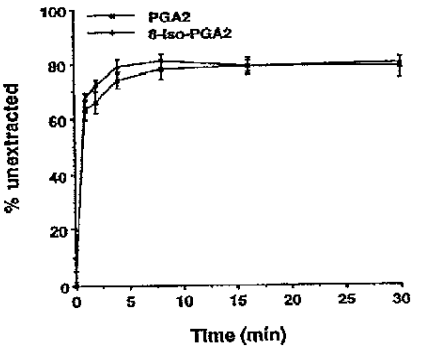

FIG. 8. **Time course of GSH-catalyzed conjugation of 15-A_{2t}-IsoP and PGA_2 with GSH.** Formation of polar GSH conjugates was monitored over time and is expressed as the percent of total radioactivity that did not extract into methylene chloride.

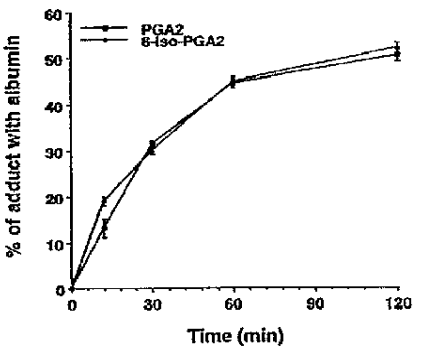

FIG. 9. **Time course of covalent adduction of 15-A_{2t}-IsoP and PGA_2 with albumin.** Formation of the adducts was monitored over time and is expressed as the percent of total radioactivity present in the protein pellet following precipitation with cold ethanol.

determining the percent of radioactivity that did not extract into methylene chloride at pH 3. Approximately 70% of 15-A_{2t}-IsoP had conjugated with GSH within 2 min, and the conjugation was complete by 8 min (Fig. 8). The time course for the conjugation of PGA_2 with GSH was found to be essentially identical. In the absence of glutathione *S*-transferase, no appreciable conjugation occurred with either compound over the same time period (data not shown). Similarly, the time course for the formation of covalent adducts with albumin was essentially identical for both PGA_2 and 15-A_{2t}-IsoP (Fig. 9).

Following administration of CCl_4 to rats, plasma concentrations of non-esterified F_2-IsoPs and D_2/E_2-IsoPs increase to very high levels (25, 27). However, we unable to detect CP-IsoPs in the circulation of rats following administration of CCl_4 in the experiments described above. We hypothesized that this may be due to rapid formation of polar conjugates, *e.g.* with GSH, following the hydrolysis of the compounds from phospholipids. To test this hypothesis, we infused a tracer quantity of radiolabeled 15-A_{2t}-IsoP into a normal human volunteer and determined the percent of radioactivity excreted into the urine that did not extract into methylene chloride. Approximately 95% of the radioactivity recovered in the urine was excreted during the first 4 h following the infusion, representing ~30% of the total amount of radioactivity infused. Only ~5% of the radioactivity present in the urine was recovered in the organic extract, suggesting that all or almost all of the compounds were present in the form of a polar conjugate(s).

DISCUSSION

We report the finding that PGA_2- and PGJ_2-like compounds are formed *in vivo* as products of the IsoP pathway. Important and interesting was the finding that these compounds not only were detected in abundant quantities esterified in rat livers following induction of an oxidant injury, but were also present in readily detectable quantities in the livers of normal rats.

The discovery of the formation of CP-IsoPs opens up numerous new avenues for scientific inquiry. The reactive nature of these compounds conferred by the α,β-unsaturated carbonyl moiety that characterizes these molecules provides a basis for hypotheses regarding their potential role in the pathogenesis of oxidant injury. As mentioned previously, CP prostanoids exert unique biological effects. They inhibit cellular proliferation via their ability to modulate a variety of growth-related and stress-induced genes, induce apoptotic cell death at higher concentrations, and activate peroxisome proliferator-activated receptor-γ (3–11). The reactive α,β-unsaturated carbonyl seems essential for many of these biological actions (15). In this regard, one might anticipate that all of the CP-IsoPs might exert similar biological effects, lessening the potential importance of elucidating the precise structures of individual compounds in the mixture. As shown in Fig. 8, PGA_2 and 15-A_{2t}-IsoP covalently adduct to protein. Interestingly, however, in intact cells, PGA_2 seems to preferentially bind to particular proteins, the functions of which remain to be elucidated (35). As shown in Fig. 7, PGA_2 and 15-A_{2t}-IsoP also rapidly undergo glutathione *S*-transferase-catalyzed conjugation with GSH. We have previously shown that Δ^{12}-PGJ_2 also rapidly conjugates with GSH, but does require catalysis by glutathione *S*-transferase (14). However, we (36) and others (37) have shown that conjugation with GSH acts to prevent the ability of CP-PGs to inhibit cellular proliferation and to induce apoptosis. Therefore, much remains to be known about the underlying molecular mechanisms that mediate the biological effects of CP prostanoids. Furthermore, the breadth of our understanding of the biological actions of CP prostanoids is probably far from complete. For example, virtually nothing is known about the biological consequences of the adduction of these molecules with DNA in regards to their potential mutagenicity.

Dehydration of PGE_2 and PGD_2 occurs in physiologic buffers, but has also been shown to be catalyzed by plasma and albumin and more recently also by human semen (21, 28, 38, 39). Plasma components could be involved in promoting dehydration of E-ring and D-ring IsoPs following their release from tissue phospholipids into the circulation. However, this study, we identified the presence of CP-IsoPs esterified in tissue membrane lipids. Whether there are also factors in cellular membranes that promote the dehydration of D_2/E_2-IsoPs remains to be explored. Nonetheless, identification of factors that may promote this dehydration may reveal potential avenues for

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intervention to modulate the formation of CP-IsoPs *in vivo*.

Our observation that 15-A_{2t}-IsoP undergoes extensive conjugation *in vivo* in humans is consistent with our previous findings that Δ^{12}-PGJ_2 also undergoes extensive conjugation in rats (14). These observations have potentially very important implications. This may explain the difficulty in the past in demonstrating the formation of CP-PGs *in vivo* because efforts were focused on detecting these compounds in free form. Even if detected in free form in small amounts, *e.g.* in urine as reported by Hirata *et. al* (19), this could potentially lead to the erroneous conclusion that the amounts formed *in vivo* may not be biologically relevant. However, quantification of the conjugated form(s) of CP-IsoPs in plasma and/or urine may provide an approach that more accurately assesses the magnitude of endogenous production of both CP-PGs and CP-IsoPs. In the case of CP-IsoPs, it is possible to assess their formation esterified in tissues of interest, but this is not amenable to human investigation. More important, as well, measurement of a conjugate of CP-PGs and CP-IsoPs would completely eliminate the confounding problem of artifactual formation *ex vivo* of what is being measured. This clearly provides the impetus for future studies aimed at elucidating the nature of these conjugates.

In summary, we have reported the discovery of a new class of reactive products of lipid peroxidation that are formed *in vivo* via the IsoP pathway. This provides a rational basis to explore in depth the biological activities of these novel molecules that may provide new insights into the biological consequences of their formation as it relates to the pathobiology of oxidant injury.

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Addendum—Since submission of the manuscript, we carried out a study to determine if 15-A_{2t}-IsoP (8-iso-PGA_2) is one of the CP-IsoPs that is produced *in vivo*. We found that a single compound isolated from the livers of CCl_4-treated rats coeluted with radiolabeled 15-A_{2t}-IsoP through four high resolving HPLC purification procedures, indicating that 15-A_{2t}-IsoP is in fact one of the CP-IsoPs formed *in vivo*. Furthermore, the data indicated that the first two HPLC procedures eliminated all other CP-IsoPs. This suggests that the mass spectrum of the compound shown in Fig. 4 that coeluted with radiolabeled 15-A_{2t}-IsoP through two HPLC purification procedures is likely a mass spectrum of 15-A_{2t}-IsoP.

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